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Effect of testosterone blockers on male aggression, song and parental care in an arctic passerine, the Lapland longspur (*Calcarius lapponicus*)

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ABSTRACT

In many passerine birds, testosterone stimulates song and aggression but inhibits paternal care, but few studies have explored whether such effects can be reversed with testosterone blockers. We explored the effect of testosterone blockers on song, aggression and paternal care of Lapland longspurs (*Calcarius lapponicus*), an arctic passerine with a short breeding season. Twenty-one "blocker males" received implants containing an androgen receptor blocker and an aromatase inhibitor, compared to 27 control males with empty or no implants. Song, aggression and other behaviors were evaluated with simulated territorial intrusions (STI) during mate-guarding, and with focal observations (without STI) during mate-guarding and incubation. Nests were monitored and nestlings weighed as an indirect measure of paternal care. During STI, blocker males exhibited similar song rates, significantly lower aggression, and were significantly less likely to be found on territory than control males. Focal observations revealed no differences in spontaneous song, aggression, foraging, preening, or flight activity. Blocker males' nestlings had greater body mass on day 5 after hatching, but this difference disappeared by fledging, and both groups fledged similar numbers of young. Two blocker males exhibited unusual paternal care: incubation and brooding of young, or feeding of nestlings at another male's nest. In sum, testosterone blockers affected aggression but not song, contrasting with results from previously published testosterone implant studies. Effects on paternal care were concordant with testosterone implant studies. These patterns may be related to rapid behavioral changes characteristic of the short breeding season of the Arctic.

In males of seasonally breeding bird species, testosterone (T) tends to promote territorial aggression and courtship, usually at the expense of paternal care (reviewed in Lynn, 2008; Vleck and Vleck, 2011). These relationships have been extensively explored in temperate-breeding passerine birds, which typically have a marked elevation in T at the beginning of the breeding season, a decline once the first brood has hatched, and subsequent elevations if new nests are initiated (Wingfield and Farner, 1978a, 1978b, 1993; Wingfield and Moore, 1987). Experimental elevation of T via implants in these temperate-zone species classically causes a resurgence of both male-male territorial aggression and singing, while inhibiting parental care (reviewed in Lynn, 2008). Curiously, testosterone blockers do not have exactly opposite effects, often decreasing song but with minor-to-no effects on male-male aggression (reviewed in Apfelbeck et al., 2013). Interpretation of these conflicting findings has been difficult due to the fact that the two experimental approaches (T implants and testosterone blocker implants) have rarely been explored in the same avian species and rarely focus on the same behavioral measures (reviewed in Apfelbeck et al., 2013, Lynn, 2008, 2016; see also Moore et al., 2018, Wingfield et al., 2014 for discussion of tropical species).

In addition, several temperate-zone avian species exhibit male "behavioral insensitivity" to T implants, i.e., males do not respond to T implants with the predicted increase in song and aggression, nor with the predicted decrease in parental care (Lynn, 2008). Such behavioral insensitivity to T has been interpreted as adaptive for "short season" reproduction (i.e., lack of breeding opportunities after the first nest, common in high latitudes) and/or essential paternal care (i.e., male parental care may be essential for normal nestling growth and fledging success) (reviewed in Lynn, 2008, 2016). Either of these traits may reduce the adaptive value of maintaining androgenic influences on reproductive and territorial behavior after the first nest is initiated. T-implant experiments on arctic passerines — short-season breeders that may or may not have essential paternal care — have therefore been especially informative for this field of study, and have revealed that

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several arctic passerines are behaviorally insensitive to testosterone. However, no published data exist on the behavioral or physiological effects of testosterone blockers in these arctic species. Here we present data from the 1990s on the effect of blockers on behavior in an arctic passerine with a short breeding season, the Lapland longspur (Hunt et al., 1999; Hunt and Wingfield, 2004).

The Lapland longspur (*Calcarius lapponicus*) is a socially monogamous species with a circumpolar range, breeding almost exclusively on arctic tundra. Studies of Lapland longspur breeding phenology in the 1990s at Toolik Field Station, Alaska, revealed that at that time, only ~30 days elapsed from first arrival of Lapland longspur adults to fledging of young from the nest (Hunt et al., 1995, 1997). Males progressed rapidly and near-synchronously through several behavioral stages: a brief 1–2 day "song display" stage characterized by high territorial display flights, followed by ~5 days of mate-guarding, ~12 days of incubation and ~10 days of feeding nestlings. Males have not been reported to incubate eggs, but do feed young, and paternal care is essential for normal nestling growth (Hunt et al., 1999). Male song is most common in the display stage, while overt male-male aggression (threat posture, attacks) peaks during the mate-guarding stage (Hunt et al., 1997). Throughout the breeding season, testosterone correlates more closely with song than with aggression (Hunt et al., 1997). This species has been classified as partially insensitive to testosterone due to the fact that though T implants increase singing, they do not increase male-male aggression (Hunt et al., 1997; Lynn, 2008, 2016). Additionally, T implants have only a minor and transient influence on paternal care, with no effects on final fledgling mass or nest success (Hunt et al., 1999; Lynn, 2008, 2016).

In this study, effects of testosterone were experimentally inhibited via administration of an anti-androgen and an aromatase inhibitor (together, "blockers") to intact free-living males. An aromatase inhibitor was included due to the fact that some behavioral effects of T in male birds are mediated via aromatization of T to estradiol (Schlinger and Callard, 1990; Soma, 2006). Primary questions were (1) whether blockers affect male-male aggression and/or song, evaluated via simulated territorial intrusions (STIs) during mate-guarding and with focal observations during both mate-guarding and incubation; and (2) whether blockers increase parental care, assessed indirectly via nestling mass and nest success. Our predictions, based on patterns seen in parallel T-implant studies (Hunt et al., 1997, 1999), were that blockers would reduce song but would have no effect on aggression, and would mildly enhance paternal care.

1. Methods

1.1. Field site and general methods

Lapland longspurs were studied within the Research Natural Area of Toolik Field Station ("Toolik," North Slope Borough, Alaska, 68° 38′ N, 149° 38′ W) in May and June of 1992 and 1994. (Funding constraints prevented publication at the time of the study.) At the time of this study, Lapland longspurs at this site had never been observed to fledge two successive broods, and re-nesting (initiation of a new nest upon failure of the first nest) had only been observed if the first nest failed before June 9th (Hunt et al., 1995, 1997, 1999; Hunt and Wingfield, 2004; see also Custer and Pitelka, 1977, for renesting reports from other sites).

Forty-eight males were captured during the first few days of their arrival on the breeding grounds (i.e., "display phase" of Hunt et al., 1995) in Potter traps baited with seed, and each bird was assigned a unique combination of colored leg bands and a numbered U.S. Fish & Wildlife Service aluminum band. Ninety-four percent (45 of 48) of implanted birds were subsequently recaptured during later trapping efforts and/or during STI, with one blocker bird and two empty-implanted birds not recaptured. At each capture, any scheduled blood samples were taken (see below) after which body mass and fat stores were evaluated and birds were examined for general health, position of any implants, clinical status of implant incision sites, and evidence of molt. Body mass was measured to the nearest 0.1 g using a Pesola spring scale, and furcular and abdominal fat stores were scored on a scale from 0 to 5 (as in Wingfield and Farner, 1978b, with 0 = no visible fat and 5 = bulging fat deposits). For implanted birds (see next), body mass and fat stores were evaluated both before and after birds received implants. The "pre-implant" measurements are from the display phase when the bird was first captured, while "with-implant" measurements were taken 7.25 ± 0.54 d later (mean ± SEM) during the mate-guarding stage. Dates of capture ranged from 24 May–29 June in 1992, and from 14 May–30 June in 1994. Time of capture ranged from 5:30 am to 11:30 pm in both years; note that this field site is under 24 hr daylight during the dates of the study.

All experiments were approved at the time of the study by the University of Washington's Institutional Animal Care and Use Committee (protocol #2212-22, "Hormone-behavior interactions in arctic birds"), and followed all applicable local, state, and federal guidelines.

1.2. Blocker implants and empty (control) implants

The forty-eight males were assigned at capture in random order either to a blocker group ("blocker" group) or a control group. Blockers (ATD + flutamide) were given to a total of 21 males (eleven in 1992 and ten in 1994), referred to as "blocker birds." For implanting, birds were anesthetized with a light dose of the inhalant methoxyflurane (Metofane; Pitman-Moore) and 2 mm incisions were made on the left and right flank above the knees. Implants were gently inserted through these incisions so that they rested subcutaneously along the dorsal portion of the ribcage, after which the incision site was sealed with a veterinary skin bond (Nexaband; Tri-Point Medical, Raleigh, NC, USA). The left flank implant contained ATD (1,4,6-androstatriene-3,17-dione; Steraloids, Inc., Newport, RI, USA), which is an irreversible aromatase inhibitor (Brodie et al., 1982; Neri and Peets, 1975). The right flank implant contained the nonsteroidal anti-androgen flutamide (a-a-a-trifluoro-2-methyl-4′-nitro-m-propionotoluidide, Schering Corporation, Kenilworth, NJ, USA), a selective androgen-receptor antagonist (Labrie, 1993). This combination of ATD and flutamide has been shown to be effective in reducing androgen-dependent behaviors in many bird species (e.g., Adkins-Regan and Leung, 2006; Berzins et al., 2018; Canoine and Gwinner, 2002; Hau et al., 2000, 2004; Shevchouk et al., 2018; Small et al., 2015; Van Duyse et al., 2005). Each implant consisted of crystalline ATD or flutamide packed into a 12 mm length of Silastic tubing (i.d. 1.47 mm, o.d. 1.96 mm). Implants were sealed at both ends with Silastic medical adhesive (Dow-Corning, Auburn, MI, USA); the sealant reduces the effective length of the implant by 1 mm at each end. Prior studies have shown that ATD and flutamide diffuse slowly across the walls of this size of Silastic implant, with an estimated release rate of ~8 mg of ATD/kg/d and ~8 mg of flutamide/kg/d over at least 30 d (Beletsky et al., 1990; Hau et al., 2000; Schwabl and Kriner, 1991; Soma et al., 1999b).

Empty Silastic implants were given to sixteen males (eight in 1992 and eight in 1994). An additional eleven unimplanted males (five in 1992 and six in 1994) were also studied. As has been demonstrated in several prior studies on this species at this site (Hunt et al., 1995, 1997, 1999), the empty-implanted birds and unimplanted birds in this study showed no obvious or statistically significant differences in any physiological, endocrine or behavioral measures (data not shown), and were therefore combined into a single "control group" of 27 males. The control group was studied for hormones, behavior, and nestling mass. Some nest success data were contributed from three additional nests of unbanded males (i.e., males that were never caught); that is, for nest success, all known Lapland longspur nests were monitored regardless of banding status of male. This was done in order to increase sample size of nests that survived to fledging, in light of known high rates of nest

depredation at this site (Hunt et al., 1997, 1999). At the end of the study, attempts were made to recapture all birds for implant removal, but it proved difficult to recapture males at this time. Implants were successfully removed from a total of six blocker males and three control males.

Effectiveness of blocker implants was assessed by comparing post-implant plasma T and luteinizing hormone (LH) concentrations in blocker birds vs. control birds. A combination of anti-androgens and aromatase inhibitors will usually cause an increase in endogenous T and LH, due both to the inhibition of aromatization as well as reduction of negative feedback at the pituitary and hypothalamus (Bhatnagar et al., 1992; Hau et al., 2004). To eliminate potential effects of STIs on circulating T, this analysis was restricted to nine blocker males that were recaptured while foraging at seed-baited Potter traps (i.e., not captured during an STI). If multiple such samples were available from the same bird, we selected the sample closest to 10 d after implant, to ascertain whether implants were still effective during incubation-stage simulated territorial intrusions, typically performed approximately 10 d after implanting. Due to the rapid changes of testosterone concentrations with calendar date in this species, available comparison samples from control males (i.e., also captured while foraging) were selected to match the blocker samples for date, resulting in a sample size of nine control birds for this comparison. All nine blocker birds had known territories and six had known nests; eight of the nine control birds had known territories, and at least five had nests (likely seven, as indicated by typical "sentry" behavior; Hunt, pers. obs.). Samples were analyzed for T and LH as described below.

1.3. Blood sampling and immunoassay

Birds were sampled for blood upon initial capture (unless implanting occurred on the same day) and on any subsequent captures that occurred at least seven days after any previous blood sampling event. The purpose of blood sampling was to monitor endocrine state of birds prior to implanting, and effectiveness of implants after implanting. Because acute stress may affect testosterone levels within approximately 10 min (Deviche et al., 2010, 2012; Wingfield et al., 1982), we monitored traps continuously and sampled all birds within 10 min of capture. Heparinized microhematocrit tubes were used to draw up 250–300 μL of whole blood from a small puncture in the alar vein. Blood samples were stored temporarily in small field coolers and centrifuged within 6 h, with plasma transferred to cryovials and stored at −20 °C until assay.

Testosterone was assayed with an in-house ^{3}H radioimmunoassay (RIA) as described in Ball and Wingfield (1987), modified as in Wingfield et al. (1991). Briefly, 20 μL of radiolabelled testosterone was first added to every sample to track percentage recovery, followed by extraction of steroids from plasma with distilled dichloromethane and separation on diatomaceous earth/glycol micro columns. The testosterone fraction was collected and a subsample assessed for radiolabel content (to determine percentage-recovery for each sample) followed by RIA of the remainder. Bound and non-bound fractions were separated with dextran-coated charcoal, and the bound fraction was then mixed with Ultima Gold scintillation fluid (Packard Instruments, now PerkinElmer, Waltham, MA, USA) and counted 10 min in a Beckman-Coulter scintillation counter.

Remaining plasma was assayed for luteinizing hormone (LH), using a post-precipitation, double-antibody RIA as described in Follett et al. (1972, 1975), modified as in Wingfield et al. (1991). Four blocker samples had insufficient plasma volume for LH assay, and therefore sample sizes for LH were restricted to five blocker samples and five control samples matched for date.

In both assays, all standards, samples and controls were assayed in duplicate with results averaged, and with final results corrected for percentage-recovery. Samples were processed in two testosterone assays and one LH assay, and blocker and control samples were evenly divided across the two testosterone assays. Intra-assay variation was 11% for the testosterone assay and 9% for the LH assay; inter-assay variation was 4% for the testosterone assay; and mean recovery percentages were 70%. Both assays have previously been used successfully in a wide variety of passerine birds (e.g., testosterone: Ball and Wingfield, 1987, Hunt et al., 1995, 1997, 1999; Schoech et al., 2013; LH: Wikelski et al., 2000, Wingfield and Farner, 1978b) and both demonstrate good parallelism with plasma of this species (data not shown).

1.4. Simulated territorial intrusions (STIs)

Territorial aggression was assessed with STIs and focal observations. Our STI protocol for Lapland longspurs, and the ethogram used to score behaviors, are described in detail in Hunt et al. (1995, 1997). Briefly, a live caged "decoy" bird was placed on the resident male's territory, along with a speaker broadcasting conspecific local song. If the male had a known nest, the STI was placed 20 m from the nest; if there was no known nest, the STI was placed in the center of the area over which that male had been observed to do song flights (high territorial display flights with song), i.e., the center of his presumed territory.

STIs were attempted on 18 blocker birds and 15 control birds, all assessed during mate-guarding. Dates of STIs spanned June 2–8 in 1992 and June 1–5 in 1994. STIs and other behavioral observations occurred between 06:00 and 21:00 (avoiding the behaviorally quiescent period between 12:00 am-5:00 am in this species under 24 h natural light; Ashley et al., 2013), using approximately alternating order between blocker birds and control birds. There were no significant differences between groups in time of day of observations (data not shown).

Lapland longspur males sometimes forage far off-territory, and therefore once an STI attempt began (playback started, decoy uncovered), behavioral observations did not begin until the male could be confirmed to be on territory. Males were classed as "not found" if they were not seen or heard on their presumed territory after 30 min of searching (i.e., observer continuously scanning a ~100 m diameter tundra territory, listening for vocalizations, and walking in a zigzag pattern to flush any sitting birds). Once a male was located, behavioral observations began and continued for 10 min. All birds in this study were naïve to STI, and all were presented with the same STI recording, a natural sequence of several songs from a single local male recorded in 1991. Later studies on this population revealed no significant differences in behavioral responses to STI when using songs from different local males or from non-local males with different song dialects, and no response to heterospecific song of Gambel's white-crowned sparrow, *Zonotrichia leucophrys gambelii* (Hunt, unpublished data).

During the 10 min of observations, the male's estimated distance from the decoy, along with any postural changes, songs or aggressive actions (see next) were narrated continuously into a handheld recorder by a single trained observer blind to the experimental status of each bird. Once the 10 min of observation concluded, two Potter traps by the decoy were opened and baited with seed in an attempt to capture the bird for body mass and fat measurements, and to verify presence and correct placement of implants. For all birds, identity was confirmed via color bands during the STI. Recordings were later transcribed to score occurrence and timing of four behaviors (following Hunt et al., 1997): (1) Number of songs during the ten minutes. Because of lack of correlation between song and physical aggression in this species (Hunt et al., 1995, 1997), song was considered primarily a courtship signal rather than an indicator of aggression. (2) Time within 5 m, total time (out of 10 min) that the resident male spent within 5 m of the decoy, interpreted as a measure of interest but not necessarily of aggression. (3) Threat posture, total time (out of 10 min) that the resident male performed a characteristic posture with the body held horizontally, bill tilted up, and wings slightly drooped, as in Drury (1961); previous studies show that this posture correlates with physical attacks (Hunt et al., 1997). (4) Number of attacks, defined as striking of the decoy's

cage with bill or feet. The latter two behaviors were both considered indicators of male-male aggression. Following Hunt et al. (1997, 1999), these two behaviors were combined into a single relative "aggression score," as follows: each bird was ranked against all other birds for time spent in threat posture, and was separately ranked for number of attacks, with both datasets ordered low-to-high. The relative aggression score is the sum of the two ranks, with a lower score indicating less aggression.

1.5. Focal observations

Focal observations were used to examine spontaneous rates of song and aggression, and also to assess potential nonspecific side effects of blocker implants on non-territorial behaviors (e.g., flying, preening, foraging). Eight blocker males and eight control males that all had active nests were scheduled for two focal observations, one during mate-guarding and one during incubation. Dates of focal observations spanned June 1–12 in 1992, and June 1–11 in 1994. By the time of incubation, sample sizes reduced to six blocker males and six control males due to failure of some nests (depredation or abandonment). During an attempted focal observation, birds were classed as "found" or "not found" as above, after 30 min of searching their presumed territory. "Found" males were then observed for 30 min from a distance of approximately 30 m, with the observer continuously narrating into a recorder any songs, flights, preening, and foraging, and any interactions with conspecific females or other conspecific males. Previous studies indicate that a 30 min observation period is a sufficient duration to capture almost all behaviors of interest (Hunt et al., 1995, 1997). All focal observations were performed by a single observer blind to the experimental status of each bird. Recordings were later transcribed to score occurrence and timing of the following behaviors, following Hunt et al. (1995, 1997): (1) number of songs/h, (2) number of "song flights"/h, defined as any flights that included song (territorial display flights, typically also with steep take-off angle of $> 30^{\circ}$ and characteristic circling flight pattern high over the territory), (3) number of "low flights"/h, defined as flights with no song (typically also with low take-off angle of $< 20^{\circ}$ and no circling), (4) % min (percentage of observation minutes) with any foraging (slow walking with head down and frequent pecking at ground or vegetation), (5) % min within 10 m of a female, (6) occurrence of any preening (scored yes/no), and (7) occurrence of any aggression, defined as any threat posture or attacks (scored yes/no).

1.6. Nest success & nestling growth

As an indirect measure of quality of paternal care (see Hunt et al., 1999; Hunt and Wingfield, 2004), eight blocker nests and ten control nests were checked daily for number of eggs or nestlings, with every nestling weighed individually once per day, between 12:00 pm and 6:00 pm, with a portable digital scale accurate to 0.01 g. The individual masses from all nestlings at a given nest were averaged to produce a mean nestling mass for that nest on that day. An additional 20 nests of control males were also monitored for number of eggs, hatchlings and fledglings.

Lapland longspur nestlings typically fledge on days 9–10 after hatch, but can fledge as early as day 7 if disturbed (McLaughlin and Montgomerie, 1985, 1989; Williamson, 1968a, 1968b). Successful fledging was assumed to have occurred if the nest was first found empty on day 7 or later (where day 0 = day that the first hatchling is found in the nest), with the nest lining undisturbed. Nests were classed as "depredated" if nestlings disappeared before day 7 (regardless of nest appearance), or if nestlings disappeared on or after day 7 and the nest was disturbed (down lining pulled out, grass cup torn up, and/or disappearance of any unhatched eggs). Nests were classed as "abandoned" if eggs never hatched, nest lining was undisturbed with eggs cold, and a blade of grass placed across the nest remained undisturbed for at least 24 h. Occasionally single nestlings were found dead, but with the nest intact and with other nestlings all surviving; this was classed as "single nestling death".

1.7. Statistical analyses

Two-group comparisons of normal continuous data (testosterone concentrations, body mass, fat scores, nest clutch size, #hatchlings, #fledglings, nestling masses) were performed with Student's *t*-test. Two-group comparisons of non-normal continuous data (LH data and all behavioral data) were performed with nonparametric Mann-Whitney tests. A multiple-comparisons correction was employed for aggression-related measures that drew from the same dataset (aggression score, threat posture and attacks) using the false-discovery-rate method, e.g., related univariate analyses were first ranked by *P*-value (lowest to highest) with each *P* value then multiplied by [number of analyses]/rank (adapted from Curran-Everett, 2000, 2004, to allow comparison to a single unchanged alpha of 0.05). Proportion analyses (e.g., proportion of birds that sang or didn't sing; found / not found data; proportion of nests depredated or abandoned) were analyzed with Fisher exact tests. Nest success was evaluated via comparison of clutch size, hatching success (# of live hatchlings per nest), fledging success (# of young fledged per nest), and causes of nest failure (depredation, abandonment, single nestling death). Hatching and fledging success were also evaluated as percentages (% of eggs that produced live hatchlings, % of hatchlings that fledged), with no changes in significance or direction; only the absolute nest success data are presented here. Nestling growth rates were evaluated with repeated-measures ANOVA from days 0–7. Due to failure of some nests before day 7, nestling mass was also compared via Student's *t*-test for day 5 only, the inflection point of the nestling growth curve in this species (i.e., day of fastest nestling growth) and the first day when females can no longer fully compensate for reduced care by the male (Hunt et al., 1999; Hunt and Wingfield, 2004). Means ± SEM are reported for both normal and non-normal data to enable comparison to other published literature, while medians and ranges are also reported for non-normal data. For any statistically significant differences between groups ($P < 0.05$), effect sizes were estimated as follows: bias-corrected Hedge's *g* for two-sample parametric comparisons (Hedges, 1981); Pearson's *r* for two-group nonparametric comparisons (Cohen, 1988; Tomczak and Tomczak, 2014); and relative risk (RR) for 2 × 2 contingency tables (Field, 2013). Statistical analyses were performed with Instat 3 and Prism 7 for Macintosh. All tests were two-tailed with alpha set at 0.05.

2. Results

2.1. Hormones, body mass and fat scores

After birds received implants, testosterone was significantly higher in blocker birds than control birds (blockers birds, 2.6 ± 0.6 ng/mL; control birds, 1.0 ± 0.4 ng/mL; $t_{16} = 2.19$, Hedge's $g = 1.00$, $P = 0.04$). Blocker and control birds did not differ significantly in LH concentration, though sample size was smaller for this comparison (blocker birds, 1.4 ± 0.5 ng/mL, range 0.2–3.4 ng/mL, median = 1.2 ng/mL; control birds, 1.6 ± 0.2 ng/mL, range 1.0–2.1 ng/mL, median = 1.7 ng/mL; $U = 18$, $Z = 1.15$, $P = 0.25$). Statistical power was low for the latter comparison (estimated < 50% probability of detecting a hypothetical 30% difference between group means).

Before receiving implants, blocker birds had slightly lower body mass than control birds (blocker birds, 25.8 ± 0.3 g; control birds, 27.5 ± 0.6 g; $t_{46} = 2.25$, Hedge's $g = 0.80$, $P = 0.03$) but had similar fat scores (blocker birds, 1.00 ± 0.2; control birds, 1.35 ± 0.2, $t_{46} = 1.24$, $P = 0.22$). However, when comparing only those birds re-sighted later for behavioral study (STIs and/or focals), there was no difference in pre-implant body mass (blocker birds, 25.9 ± 0.4 g;

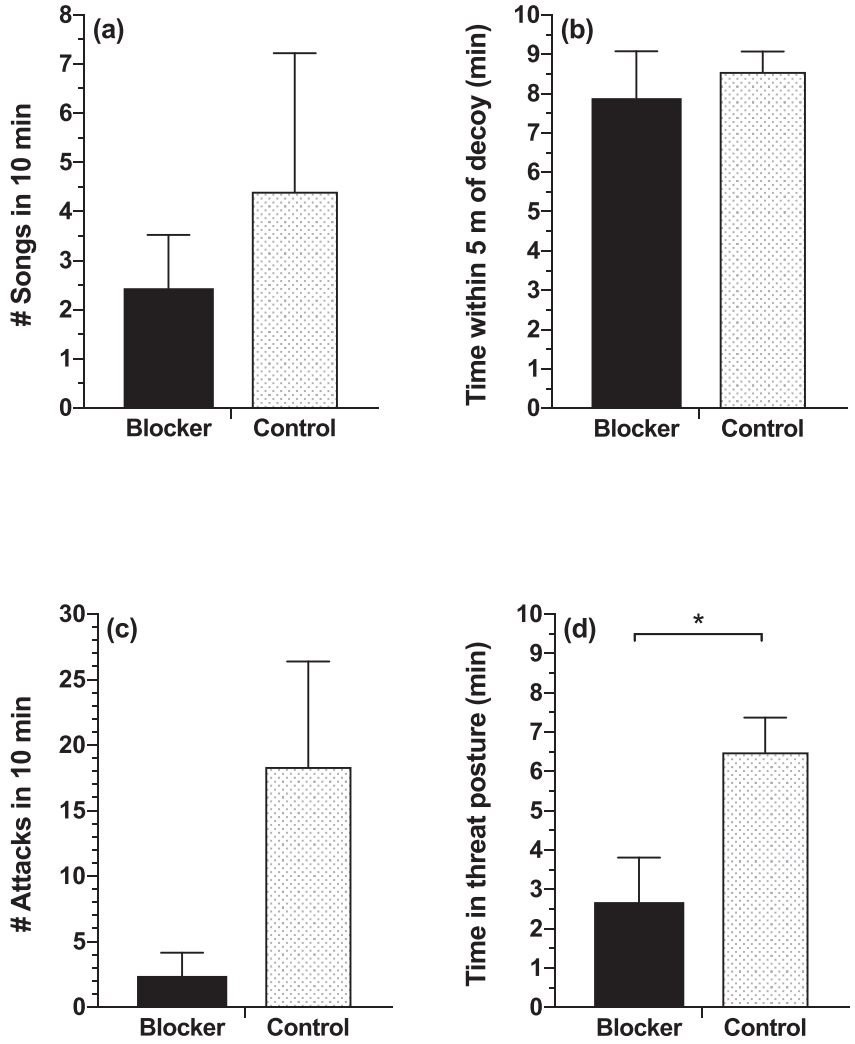

Fig. 1. Behavioral responses to simulated territorial intrusions presented to blocker-implanted and control male Lapland longspurs during mate-guarding. Bars indicate mean ± SEM; sample sizes were $n = 10$ blocker birds and $n = 15$ control birds. Asterisk = significantly different at $P < 0.05$.

control birds, 26.7 ± 0.4 g; $t_{21} = 1.48$, $P = 0.16$). Birds captured with implants, i.e., during mate-guarding, showed no significant differences in with-implant body mass or with-implant fat scores compared to controls (mass: blocker birds, 27.4 ± 0.4 g; control birds, 27.0 ± 0.2 g; $t_{39} = 1.06$, $P = 0.30$; fat: blocker birds, 0.8 ± 0.2; control birds, 0.9 ± 0.1; $t_{39} = 0.41$, $P = 0.68$).

2.2. Behavioral response to STI

Significantly fewer blocker birds than control birds were found on territory for STI during mate-guarding (blocker birds, 61% [11 of 18] birds found; control birds, 100% [15 of 15] birds found; RR = 0.61, $P = 0.01$). Five of the seven "not found" blocker birds were later re-sighted on territory and at least three had active nests at the time of the STI. The voice recording device failed for one blocker STI; final STI sample sizes were ten blocker birds (eight with known nests) and 15 control birds (eight with known nests).

Behavioral data from STIs are presented in Fig. 1. Blocker birds spent significantly less time in threat posture ($U = 118$, $Z = 2.39$, $r = 0.48$, $P = 0.03$) and showed a nonsignificant trend toward fewer attacks ($U = 103$, $Z = 1.68$, $P = 0.09$). The two groups showed no apparent differences in song rate ($U = 92.0$, $Z = 0.94$, $P = 0.32$) or time within 5 m of the decoy ($U = 95$, $Z = 1.14$, $P = 0.26$). Relative aggression score was significantly reduced in blocker birds ($U = 120.5$, $Z = 2.52$, $r = 0.51$, $P = 0.03$).

2.3. Focal observations of spontaneous behavior

During mate-guarding, similar percentages of blocker birds (75%, or 6 of 8 birds) and control birds (100%, or 8 of 8 birds) were found on territory during the scheduled focal observations ($P = 0.48$). During incubation, 55% (6 of 11) of blocker birds and 92% (11 of 12) control birds were found on territory; this difference was not quite significant ($P = 0.07$). In both sets of focal observations (mate-guarding and incubation), blocker birds did not differ significantly from control birds in any measure of singing, flights, foraging, preening, time near females, or aggression (Table 1).

2.4. Nest success, nestling growth and paternal care

Blocker birds and control birds had similar nest success. Blocker birds produced (means ± SEM) 4.63 ± 0.26 eggs, 4.25 ± 0.62 hatchlings, and 3.00 ± 0.79 fledglings per nest, with no nests (of 8 total) abandoned, one nest depredated before hatching, and three cases of single nestling death. Control birds produced 4.33 ± 0.18 eggs, 2.97 ± 0.37 hatchlings, and 1.59 ± 0.35 fledglings per nest, with 3 nests (of 30) abandoned (all before hatching), 9 nests depredated (all after hatching), and 7 cases of single nestling death. There were no significant differences in any of these measures ($P > 0.05$ for all comparisons).

There were no overall differences in mean nestling mass in those

Table 1
Results of focal observations on male Lapland longspurs with blocker or control implants, observed during mate-guarding and incubation. All data were non-normal and are presented as median (range). "Song flights" are territorial display flights with steep takeoff angle that included song; "low flights" are flights with low takeoff angle and with no song, i.e., not a territorial display; "%min" = percentage of observation minutes during which the behavior occurred at least once; aggression = threat posture or physical striking of other bird. Sample sizes were $n = 8$ per group during mate-guarding and $n = 6$ per group during incubation; lower n's in incubation are due to nest failures. *Z*-values and *P*-values shown for Mann-Whitney tests on continuous data, and chi-square and *P*-values shown for Fisher exact tests on categorical data.

	Blocker	Control	Z or X^2	P
Mate-guarding:				
Songs/hr	21.87 (0.0–85.7)	29.19 (0.0–133.5)	0.21	0.83
Song flights/hr	3.57 (0.0–10.0)	8.27 (0.0–34.5)	0.96	0.34
Low flights/hr	7.29 (0.0–25.0)	5.54 (0.0–21.0)	0.16	0.87
%min within 5 m of female	70.83 (0.0–100.0)	37.54 (0.0–100.0)	0.06	0.96
%min with foraging	73.43 (14.3–90.5)	65.75 (0.0–100.0)	0.00	> 0.99
Preening	3 of 8 preened	0 of 8 preened	3.69	0.20
Aggression	5 of 8 aggressive	3 of 8 aggressive	0.25	0.62
Incubation:				
Songs/hr	30.00 (0.0–123.8)	15.00 (0.0–67.5)	0.46	0.65
Song flights/hr	2.38 (0.0–30.0	5.25 (0.0–24.0)	0.16	0.87
Low flights/hr	19.07 (6.7–30.0)	13.50 (0.0–32.9)	0.48	0.63
%min within 5 m of female	0.00 (0.0–39.1)	7.50 (0.0–40.0)	1.05	0.29
%min with foraging	79.29 (0.0–100.0)	52.50 (5.0–75.0)	0.26	0.26
Preening	1 of 6 preened	2 of 6 preened	0.44	> 0.99
Aggression	1 of 6 aggressive	0 of 6 aggressive	1.09	> 0.99

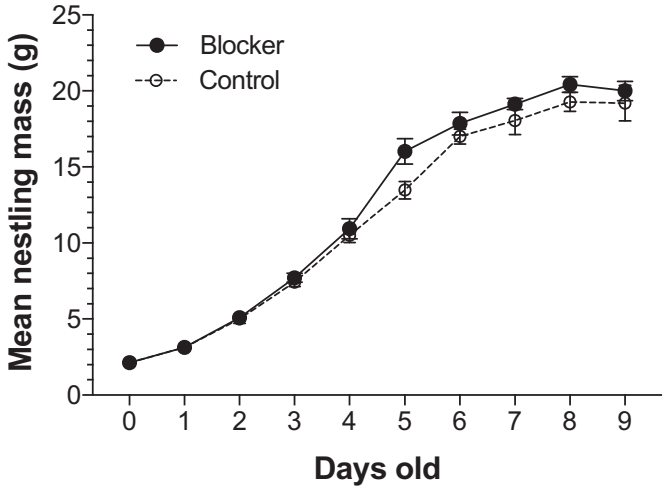

Fig. 2. Mean nestling mass in nests of male Lapland longspurs given testosterone blocker implants ("Blocker") compared to nests of empty-implanted or unimplanted males ("Control"). Data are shown as means ± SEM. Day 0 = day that first nestling was found. Sample sizes at day 0 were $n = 8$ blocker nests and $n = 10$ control nests, with subsequent losses of two of the blocker nests due to nest depredation.

nests that survived to day 7 (six blocker nests and ten control nests; $F_{1,14} = 0.52$, $P = 0.48$; Fig. 2), and mean nestling masses by the time of fledging were very similar. However, on day 5 after hatch, the day of fastest nestling growth, blocker males' nests had significantly greater mean nestling mass than control males' nests (blocker nests, 16.0 ± 0.8 g, $n = 7$; control nests, 13.5 ± 0.6 g, $n = 11$; $t_{16} = 2.60$, Hedge's $g = 1.17$, $P = 0.02$).

Two blocker males were noted to exhibit unusual parental behavior: (1) Blocker male #39165 was found sitting on eggs during a routine nest check. Due to this unusual behavior, a videorecorder was later (after eggs hatched) placed by this nest for a single 2 h recording session, during which this male was videotaped crouched in the nest brooding the nestlings, a behavior not typical for males of this species. (2) During a routine check of a control male's nest after young had hatched, blocker male #39162 was spotted carrying a mouthful of insects very nearby, i.e., #39162 was at the wrong nest. Blocker male #39162 was an immediate neighbor of the control male, and #39162's own nest had been depredated a week earlier during incubation. A videorecorder was placed at the control nest for a single 2 h recording session, and blocker male #39162 was confirmed to be regularly feeding the control male's nestlings, a behavior not typical for this species (even despite nest depredation). The control male and his female were also feeding the young regularly. The video recording revealed that the two males (identities confirmed by visible color bands in the recording) occasionally encountered each other at the nest's edge, but did not respond noticeably to each other's presence (no threat posture or attacks were noted). Each time that one male arrived with food at the nest while the other male was already feeding the young, the newly arrived male waited at the nest's edge till the other male departed. Anecdotally, all three adults attempted to defend the nest ("sentry" behavior, alarm calling, and distraction displays) whenever observers approached. (This nest's nestling mass data were excluded from the control group, due to the fact that three adults rather than two were feeding the young.)

3. Discussion

3.1. Song and aggression

Testosterone blockers reduced territorial aggression of male Lapland longspurs in response to STI (Fig. 1), with significant reductions in both threat posture and relative aggression score. Further, blocker males were significantly less likely to be found on their territories during mate-guarding, a time when most control birds are still consistently territorial. Nevertheless, song — classically the most T-dependent of all behaviors in male passerines — was unaffected, both during STIs and in focal observations. The elevated T in blocker birds indicates that the blocker implants were likely functioning as expected, and no non-specific effects of blockers were detected on low flights, foraging, preening, mass or fat scores. Our interpretation is that the blocker implants were having the desired specific effects on androgen function (though see Soma et al., 1999b). Thus, contrary to our predictions, blockers caused a consistent reduction in territoriality and male-male aggression, yet did not show the expected effect on song.

These patterns — a decrease in aggression, yet no effect on song — contrast both with findings from T implants in this same species, and also with typical effects of T implants and blockers in other (primarily non-arctic) species. Many studies in temperate-zone avian species have clarified that the typical response of a seasonally breeding male

passerine to T implants is an increase in both song and aggression (reviewed in Lynn, 2008). Blockers, though less often studied, usually cause a decrease in song but minor-to-no effects on aggression (reviewed in Apfelbeck et al., 2013, though see Soma et al., 1999b). Lapland longspurs are already known to be an outlier in their response to T implants, which in this species increase song but not aggression (Hunt et al., 1997). Our study indicates that Lapland longspurs also may have an unusual response to blockers, and that their response to exogenous T differs qualitatively from their response to blockers.

The contrasting results seen in Lapland longspurs to exogenous T (increase in song, no change in aggression) vs. blockers (no change in song, decrease in aggression) are challenging to explain mechanistically, but may be related to the different breeding stages studied. Blocker birds in this study were presented with STI during mate-guarding, while other published studies on T-implanted Lapland longspurs (in Hunt et al., 1997) examined STI responses only during incubation. Social cues may be important; for example, song during mate-guarding could be stimulated by behavioral cues from the fertile female as well as by T concentration, whereas later in incubation (when the female is no longer fertile), endocrine influences may become relatively more important. There could also be mechanistic changes in receptor density and/or aromatase activity underlying these shifts in hormone-behavior relationships. Soma et al. (1999a) found that male Lapland longspurs have rapid alterations in activity of aromatase, 5α-reductase, and 5β-reductase in multiple brain areas during the first weeks on the breeding grounds. Such changes could explain how certain behaviors could shift from being influenced by T during mate-guarding (this study) to being unaffected by T just a week later in incubation. Finally, it is also possible that neuronal circuits involved in song and in aggression may have different sensitivity to T blockers, such that greater doses of T blockers than those tested here might have different effects.

3.2. Parental care

Anecdotally, two blocker males exhibited forms of parental care that have not before been reported for males of this species — incubation of eggs and brooding of young in one case, and feeding young at a neighbor's nest in another case. The blocker male who fed young of another male's nest had lost his own nest; nevertheless, in numerous other cases of nest loss at this site observed during 1991–1995, and daily nest checks of all active nests during the entire study area in all five years (over 200 nests in total, monitored daily), no other males were observed feeding young of a neighbor's nest. It may be that these behaviors do normally occur but at very low rates. Unusual behaviors in response to hormone implants have also been seen in females of this species, e.g. begging behavior elicited by estradiol implants (Hunt and Wingfield, 2004). Whether such "unusual" behaviors are phylogenetic artifacts, normal-but-rare behaviors, and/or truly novel behaviors is unknown, but such observations raise the intriguing possibility that relatively minor shifts in timing of circulating hormones could result in not just quantitative but qualitative shifts in behavior.

Finally, T blockers caused a mild and transient, but statistically significant, increase in mean nestling mass at day 5, the inflection point of the nestling growth curve in this species and the day on which male provisioning of nestlings first becomes essential (Hunt et al., 1999). Given other studies of paternal care in this species (Hunt et al., 1999; Hunt and Wingfield, 2004), this effect is likely due to blocker males visiting their nests more often than control males. However, nestling mass normalized by the time of fledging and there were no significant differences in any measure of nest success. These results agree with prior studies on T implants in this species, which showed that exogenous T causes a transient decline in paternal care that briefly affects nestling mass, but with no effect on final fledging mass or nest success. Overall, it appears that T exerts a subtle inhibitive effect on male parental care, such that male Lapland longspurs may retain the capacity for higher rates of nest attendance, and perhaps even some qualitatively different parental behaviors, than they normally express. The inhibitory effect of T on paternal care, however, is mild, and we agree with Lynn (2008) that Lapland longspurs generally exhibit robustness of paternal care despite various attempts at altering their androgen state (i.e., T implants or blocker implants).

3.3. Comparative perspective on behavioral insensitivity to T

Behavioral insensitivity to T also occurs in several other members of the longspur/bunting clade. This clade comprises five species — three that breed exclusively in the Arctic (Lapland longspur, Smith's longspur, *Calcarius pictus*, and snow bunting, *Plectrophenax nivalis* [here including McKay's bunting, *P.* "*hyperboreus*"]), and two that breed exclusively in temperate zone grasslands (McCown's longspur, *Calcarius mccownii*, and chestnut-collared longspur, *Calcarius ornatus*) (Klicka et al., 2003). The clade includes various combinations of "short season" traits (the three arctic species) and "essential paternal care" traits (Lapland longspur and chestnut-collared longspur) and thus has been an invaluable taxon for comparative study of testosterone-behavior relationships. Two of the arctic species (Lapland longspur and snow bunting) as well as at least one of the temperate zone species (chestnut-collared longspur) exhibit partial or full behavioral insensitivity to T in terms of effects on song, aggression and/or parental care (Romero et al., 1998; Lynn et al., 2002, 2005; Lynn, 2008). The remaining two species of this clade have not yet been studied for testosterone-behavior relationships, with research focusing, rather, on the hypothalamo-pituitary-adrenal axis (e.g., Smith's longspur, Meddle et al., 2003; McCown's longspur, Lynn et al., 2003). Finally, two temperate or arctic species outside of the longspur/bunting clade have been reported to have at least partial insensitivity to T, the great tit (*Parus major*, Van Duyse et al., 2002), and Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*, Meddle et al., 2002). Progress has been made in the *Zonotrichia* clade, which spans tropical to arctic populations, at elucidating some of the neuroendocrinological mechanisms that may underlie these hormone-behavior changes (e.g., Soma et al., 1999a, 1999b; Meddle et al., 2002), but many other short-season breeders and essential-paternal-care species remain uninvestigated and could provide informative data. It is important, as well, to recognize that the concept of "behavioral insensitivity" to testosterone is explicitly in contrast to T-behavior relationships typical of strongly seasonal breeders, i.e. temperate and arctic species. Tropical species, which typically have much longer breeding seasons, commonly have different relationships of testosterone with reproductive behavior, with some species showing almost no effects of T on reproductive behavior (e.g., rufous-collared sparrow; Lynn et al., 2009; Moore et al., 2004a, 2004b; Moore et al., 2018; Wingfield et al., 2014).

3.4. Potential relationship to length of breeding season

Overall, certain members of the longspur/bunting clade may have fine-tuned T's diverse effects on song, territorial aggression and parental care to allow for rapid behavioral switching during the brief breeding season that has, until recently, been characteristic of their arctic habitat. Ongoing gradual changes in timing of the breeding season in the Arctic, along with other climate-change-related changes (i.e., increase in snow cover, later snowmelt, change in timing of insect bloom; Cherry et al., 2014; Hinzman et al., 2005; Hobbie et al., 2017; Sturm et al., 2001) may affect the nature and timing of these hormone-behavior interactions, and may eventually illuminate whether the patterns seen in arctic birds are related to short seasons per se (see reviews and discussion in Lynn, 2008, 2016). Although the trend and prediction is for snowmelt to occur earlier in the Arctic over the next century, increased precipitation is predicted as well. Some sites might accumulate more snow, which can result, paradoxically, in increased late spring snowfall events and later snowmelt at certain sites (Hinzman et al., 2005; Hobbie et al., 2017; Sturm et al., 2001). Indeed, at our study site at Toolik in

northern Alaska, the initiation of the growing season has been delayed by an average of 11 days in the 2000's as compared to the 1990s when this study was conducted, likely due to heavier snowpack in spring and more frequent occurrence of spring snowstorms (Cherry et al., 2014). Recent studies indicate that Lapland longspurs at Toolik may as a result actually delay nest initiation and suffer increased failure of first nests (Boelman et al., 2014, 2017; Chmura et al., 2018; Krause et al., 2016), in contrast to other sites with lesser snowpack where Lapland longspur phenology is advancing (Liebezeit et al., 2014). Increased failure of first nests, combined with potential extension of the end of the breeding season, could result in greater opportunities for replacement nests (re-nests) and/or in extension of the period of female fertility. Anecdotally, in 2018 at Toolik, an incubating female Lapland longspur was flushed off an active nest on July 2, which to our knowledge may be the latest-initiated nest of this species documented at this site (G. Levandoski, pers. comm.). It is unclear whether Lapland longspurs and other arctic-specialist species will have the endocrine and behavioral flexibility to adjust rapidly to these variable changes in phenology of their arctic environment. We hope that the historic data presented here will be of use as a comparison point for future studies of physiology, endocrinology and behavior of birds in the changing Arctic.

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